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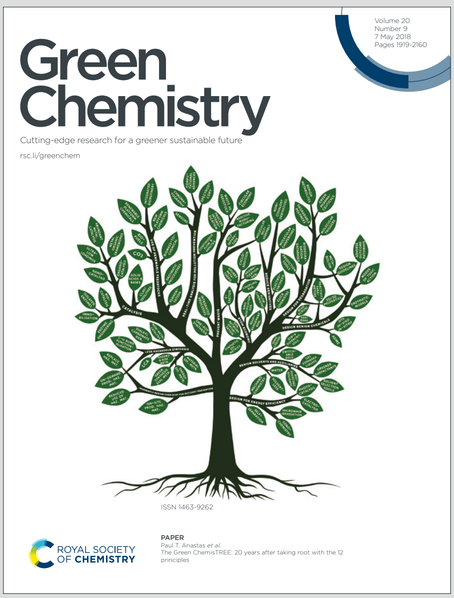

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Cleaner enzymatic production of biodiesel with easy separation procedures triggered by a biocompatible hydrophilic ionic liquid

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Abstract: The great challenges of modern industry and environment make it important to develop sustainable energy resources with low cost. In this work, a cleaner enzymatic procedure for biodiesel production was developed through the utilization of a biocompatible and hydrophilic ionic liquid [Choline][H_2PO_4]. This ionic liquid can be synthesized from cheap raw materials through simple neutralization procedures, and itself has been proven to be well biocompatible. The utilization of this ionic liquid in Novozym 435 catalyzed biodiesel production makes the reaction and work-up procedures very simple, because its hydrophilicity can lead to the implement of pseudo homogeneous reaction and then heterogeneous separation. Various oil resources such as triolein, sunflower oil and castor oil can all be converted to biodiesels with high yields. After the completion of reaction, both of the ionic liquid and Novozym 435 can be recycled and reutilized for at least five cycles without significant activity decrease. In addition, this reaction system can be convenient scaled up to multi-gram level with high efficiency and feasible separation. Overall, the above mentioned benefits make this ionic liquid based enzymatic system cleaner for the production of biodiesel and promising for the further industrial applications.

Keywords: biodiesel production; enzyme; hydrophilicity; ionic liquid; reusability

Introduction

The shortage of fossil resources and the ever increasing demands for energy have made it urgent to develop alternative energy resources with renewability, sustainability and minimum emission of greenhouse gases as compared to petroleum based diesel.[1, 2] As a promising green substitute for petroleum diesel, biodiesel is the mixture of methyl esters of long chain fatty acids (FAMEs) which is usually produced by the esterification

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of fatty acids or the transesterification of triglycerides with methanol from edible or non-edible oils and animal fats.[3-7] Compared with time and energetic consuming chemical production of biodiesel catalyzed by acid[8-10] or alkaline[11-13], enzymatic production method has received growing attention in recent years due to the mild reaction conditions, low alcohol oil ratio, no needs of pretreatment of raw materials and easy workup procedures.[14-20]

As an alternative for volatile organic solvents, ionic liquids have found extensive applications in homogeneous, heterogeneous and enzymatic catalysis procedures.[21] In biodiesel production, ionic liquids can not only efficiently overcome the inactivation of most lipases caused by methanol, but also increase the stability and activity of enzyme through suitable selection of their cations and anions.[22] The presented researches revealed that hydrophobic ionic liquids are much more favorable than hydrophilic ones because the latter may alter the conformation of protein and stripe off the essential water from lipase.[16] So, imidazolium-based ionic liquids with longer alkyl chain and hydrophobic anions such as $[C_{16}mim][NTf_2]$ and $[C_{18}mim][NTf_2]$ showed much higher catalytic activity compared with those having shorter alkyl chain and hydrophilic anions.[23] In addition, this hydrophobic ionic liquid with long alkyl chains have been believed to behave as sponge-like systems and then facilitate the reaction and separation procedures for the enzymatic production of biodiesel.[24] However, the increase in hydrophobicity of ionic liquid has significant negative effect towards aquatic environment.[25] Thus it is still a great challenge to develop cleaner reaction systems for the biodiesel production with easy operation and separation.

Very recently, Ou et al[26] found that the introduction of hydroxyalkyl moity into the structure of ionic liquids can efficiently increase the stability and activity of enzyme in ionic liquids, although their hydrophilicity is also increased. Some cholinium-based ionic liquids, which have hydroxyethyl group on the cation, have also been proved to be able to stabilize various proteins and DNA, and the existence of hydroxyethyl moity can decrease the toxicity of ionic liquids.[27] Some deep eutectic solvents (DESs) based on choline chloride have also been utilized for the production of biodiesel, but the reusability of DES and enzyme is still need to be optimized due to the relative instability of DESs in complex reaction systems.[17, 28] Therefore, in order to find excellent reusable ionic liquid reaction systems with high enzymatic activity and low ionic liquid toxicity, a series of ionic liquids composed with various cations with and without hydroxyethyl groups and anions with different hydrophobicities have been synthesized (Scheme1),

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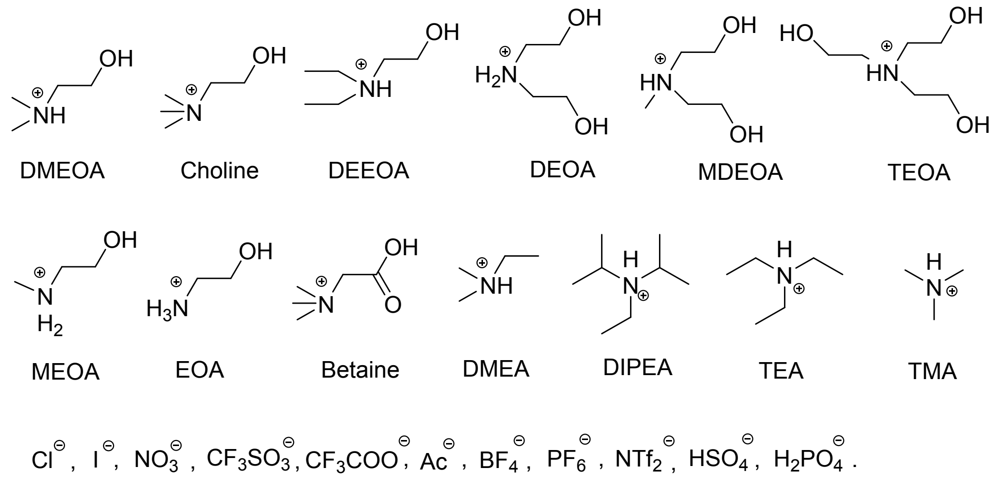
and their performances in the production of biodiesel catalytzed by Novozym 435 are investigated in this work. It is shown that both the cations and anions have significant influences on the enzymatic activity. The hydrophobic anions have no positive effect when they are combined with cations having hydroxyl groups, while the ionic liquid with nucleophilic [Cl] as anion exhibits moderate promoting effect, although the nucleophilic anions are often believed to be the inhibitor of enzyme.[29] Using the optimized ionic liquid cholinium dihydrogen phosphate, triolein and different oils such as edible sunflower oil and non-edible castor oil can all reach excellent biodesel yields under the catalysis of Novozym 435. In addition, due to the hydrophilicity of ionic liquid, the biodiesel product can be separated by simple decantation from the ionic liquid and enzyme, which makes the work-up procedure very simple, and the catalytic system including enzyme and ionic liquid can be reused as a whole for at least five cycles without significant activity decrease.



Scheme 1. The cations and anions of ionic liquids

Materials and methods

Materials

Immobilized lipase from Candida Antarctica (Novozym 435) and triolein were purchased from Sigma-Aldrich. Ethyl decanoate, methyl palmitate, methyl oleate, methyl linoleate, methyl stearate, and methyl ricinoleate were purchased from Aladdin Reagents Company. Hexane and ethyl acetate used for GC analysis were chromatographically pure and purchased from Macklin Reagents Company. N,N-dimethylethanolamine, choline, diethanolamine, N,N-diethylethanolamine, N-methyldiethanolamine, betaine, 2-(methylamino)ethanol, monoethanolamine, triethanolamine, triethylamine, N-ethyldimethylamine, N,N-diisopropylethylamine,

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trimethylamine, acetic acid, trifluoromethanesulfonic acid, trifluoroacetic acid, hexafluorophosphoric acid, tetrafluoroboric acid, bis(trifluoromethane sulfonimide), choline chloride, choline iodide, nitric acid, sulfuric acid, and phosphoric acid with AR grade were purchased from Aladdin Reagents Company. Methanol and aqueous solution for choline hydroxide were purchased from Sinopharm Chemical Reagent Co.. Sunflower oil was purchased from local supermarket. Castor oil was obtained from Macklin Reagents Company.

Synthesis of ionic liquids

Synthesis of protic ionic liquids. The protic ionic liquids were synthesized through the protonation of corresponding amines and acids [30] (Scheme 2, path (a)). In a typical reaction for the synthesis of [DMEOA][H_2PO_4], 0.1 mol N, N-dimethylethanolamine (DMEOA) dissolved in 10 ml methanol was slowly added to equal molar phosphoric acid under stirring in an ice bath for 2 h, and then the mixture was reacted at room temperature for further 24 h. The solvent was removed at 50 ^oC by rotary evaporation in reduced pressure and then dried under vacuum overnight to give the target protic ionic liquids. The other protic ionic liquids including [DEEOA][H_2PO_4], [DMEA][H_2PO_4], [DEOA][H_2PO_4], [TEOA][H_2PO_4], [MMEA][H_2PO_4], [MDEA][H_2PO_4], [Betaine][H_2PO_4], [EOA][H_2PO_4], [DIPEA][H_2PO_4], [TEA][H_2PO_4], [TMA][H_2PO_4], [DMEOA][HSO_4], [DMEA][HSO_4], [DEOA][HSO_4], [TEOA][HSO_4], [DMEOA][Ac], [DEEOA][Ac], and [MMEA][Ac] were synthesized in the same way.

Synthesis of aprotic ionic liquids. The cholinium-based aprotic ionic liquids were synthesized by the neutrilization of choline hydroxide with corresponding acids[30b] (Scheme 2, path (b)). Typically, 0.1 mol acid in 10ml water was droply added to 0.1mol choline hydroxide in aqueous solution under stirring in an ice bath for 2 hours, and then stirred at room temperature overnight. The solvent was then distilled by rotary evaporation under reduced pressure and then dried in vacuum to give the target aprotic ionic liquids.

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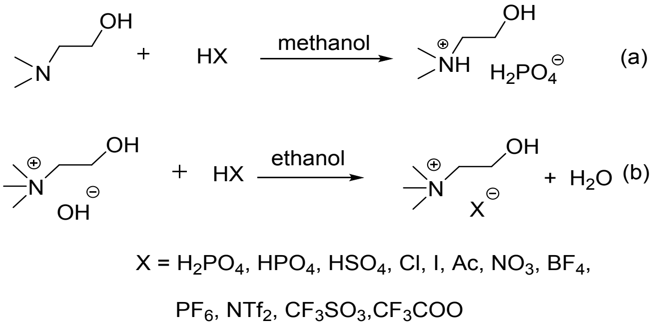

Scheme 2. Synthesis of the protic and aprotic ionic liquids.

Lipase-catalyzed biodiesel production

The transesterification reactions were performed in 10 ml reaction bottles placed in an oil bath at assigned reaction temperature. Typically, triolein (0.5 mmol, 443 mg), IL (10-100%, w/w, based on oil weight), Novozym 435 (0-15%, w/w, based on oil weight) and methanol (molar ratio of methanol to oil (M:O) 4:1-14:1) were mixed and stirred at a given temperature. After a specified reaction time (3, 6, 9, 12, 18 or 24 h), the mixture was cooled to room temperature. A certain amount of internal standard(ethyl decanoate) was added to the reaction mixture, and then 5 ml ethyl acetate was added as a solvent. After the fully mixed, 2 ml mixture was taken from the mixture and centrifuged, 50 ul solution was taken from the upper layer and then diluted to 1 ml using ethyl acetate and then, 1 μl was injected for quantitative analysis using GC-MS system.

GC-MS determination of the biodiesel yield

GC-MS analysis was carried out by using a GC-Trace 1300 (Thermo Scientifc) instrument coupled with an MS-ISQ (Thermo Scientifc) system. For the gas chromatographic analysis of sunflower oil, an Innowax column (30 m × 0.25 mm × 0.25 μm, Thermo Fisher) was used. For FAMEs identification, the following conditions were used: carrier gas (He) at 1 mL/min; inlet split ratio, 1:50; temperature program: 70 °C, 1 min, 30 °C /min; 170 ° C, 100 °C /min; and 230 °C, 7 min; MS source ionization energy, 70 eV; the scan time was 0.2 s, covering a mass range of 50-500 amu. For the analysis of castor oil, the GC was equipped with an HP-5MS column (30 m × 0.25 mm × 0.25 μm, Thermo Fisher). For FAMEs identification, the following conditions were used: carrier gas (He) at 1 mL/min; inlet split ratio, 1:50; temperature program: 80 °C, 1 min, 60 °C /min; 180 ° C, 8 min, 30 °C /min; 250 °C, 3 min; MS source ionization energy, 70 eV; the scan time was 0.2 s, covering a mass range of 50-500 amu.

The reutilization of enzyme and ionic liquid

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In the optimized reaction system, the produced biodiesel could be separated from the catalytic system by simple centrifugation and decantation when the reaction was completed. The enzyme and ionic liquid as a whole together with the un-reacted methanol and by-product glycerinum were washed with ethyl acetate for three times. The enzyme was dried at room temperature for 12 h, the ionic liquid was dried at 50 ^{o}C for 12 h, and then they could be reutilized for the next runs.

Results and discussion

Influence of the ionic liquids on the biodiesel production

The transesterification reaction between triolein and methanol was chosen to study the influence of ionic liquids on the enzymatic production of biodiesel. The results collected in Table 1 showed different ionic liquid effect compared with those reported in literatures.[16,23,29] When the anion of ionic liquids was [H_2PO_4], the ionic liquids with cations such as TMA, TEA, TEOA, EOA, MDEOA, MEOA, and betainium showed negative effect (entries 2-8, Table 1) towards the production of biodiesel compared to that with no ionic liquid was added (entry 1, Table 1), the ionic liquids with DEEOA, DMEA, DEOA and DIPEA cations showed slightly promotion for the enzyme activity (entries 9-12, Table 1), while the ionic liquids with DMEOA and Choline as cations exhibited excellent promotion effect with the biodiesel yields above 90% (entries 13, 14, Table 1). When [HPO_4] was used as anion, the effect of ionic liquids [Choline]$_2$[HPO_4] was negative but [DMEOA]$_2$[HPO_4] was a little positive (entries 15, 16, Table 1). A more acidic anion [HSO_4] (entries 17-21, Table 1) and a more basic anion [Ac] (entries 22-26, Table 1) showed different cation dependences, only ionic liquid [MEOA][Ac] was positive for the enzymatic performance. Then the other anion effects on the enzymatic production of biodiesel was also investigated using Choline as the cation (entries 27-34, Table 1), the results showed that more hydrophobic anions such as [PF_6] and [NTf_2] even have negative effect on the performances of enzyme when they were combined with cholinium cation, but the nucleophilic anion such as [Cl] and [NO_3] could give moderate promotion for the production of biodesel. The above findings fall out of the traditional relationship between ionic liquid structure and enzyme activity. The possible reason was that the ionic liquids effect towards this enzymatic reaction was performed through the synergetic effects of the cation and the anion as a whole, but not through the influences of a single ion. So, the combination of the cation and the anion of ionic liquids was superior important. The ionic liquid

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[Choline][H_2PO_4] which is hydrophilic and biocompatible have been proved to show the highest performance in promoting this enzymatic procedure. In addition, this ionic liquid is insoluble in the produced biodiesel. So, the biodiesel can be separated by simple centrifugation and decantation after the reaction, the enzyme and the ionic liquid can be reused for next cycles by simple washing and drying procedures.

Table 1. The chemical structure of the ionic liquids on the biodiesel yield

Entry[a]	IL	Yield %[b]	Entry[a]	IL	Yield %[b]
1	blank	51	19	[DEOA][HSO_4]	n.d.
2	[TMA][H_2PO_4]	40	20	[TEOA][HSO_4]	n.d.
3	[TEA][H_2PO_4]	19	21	[DMEA][HSO_4]	30
4	[TEOA][H_2PO_4]	9	22	[Choline][Ac]	7
5	[EOA][H_2PO_4]	13	23	[DMEOA][Ac]	n.d.
6	[MDEOA][H_2PO_4]	21	24	[DEEOA][Ac]	5
7	[MEOA][H_2PO_4]	46	25	[MEOA][Ac]	71
8	[betaine][H_2PO_4]	30	26	[TEOA][Ac]	16
9	[DEEOA][H_2PO_4]	80	27	[Choline][BF_4]	13
10	[DMEA][H_2PO_4]	70	28	[Choline][PF_6]	7
11	[DEOA][H_2PO_4]	73	29	[Choline][NTf_2]	5
12	[DIPEA][H_2PO_4]	73	30	[Choline][NO_3]	64
13	[DMEOA][H_2PO_4]	90	31	[Choline][Cl]	76
14	[Choline][H_2PO_4]	95	32	[Choline][I]	n.d.
15	[Choline]$_2$[HPO_4]	40	33	[Choline][CF_3SO_3]	54
16	[DMEOA]$_2$[HPO_4]	73	34	[Choline][CF_3COO]	5
17	[Choline][HSO_4]	3	35[c]	[Choline][H_2PO_4]	n.d.
18	[DMEOA][HSO_4]	n.d.			

[a]reaction conditions: triolein, 443 mg; ILs, 443 mg; lipase, 22 mg; methanol /oil molar ratio,10:1; reaction time, 24 h, 40 ^{o}C. [b]GC yield. [c]non-enzyme.

Influence of temperature on the biodiesel production

It is well recognized that the enzymatic reaction is often temperature sensitive. So, the influence of temperature on the enzymatic promoted biodiesel production was investigated from 25 to 100 ^{o}C using the optimized ionic liquid [Choline][H_2PO_4] and Novozyme 435 as catalyst. The results in Fig. 1 showed that at the temperature of 60 ^{o}C, the biodiesel from triolein could reach to quantitative yields, but further increase of

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temperature led to the deactivation of enzyme.

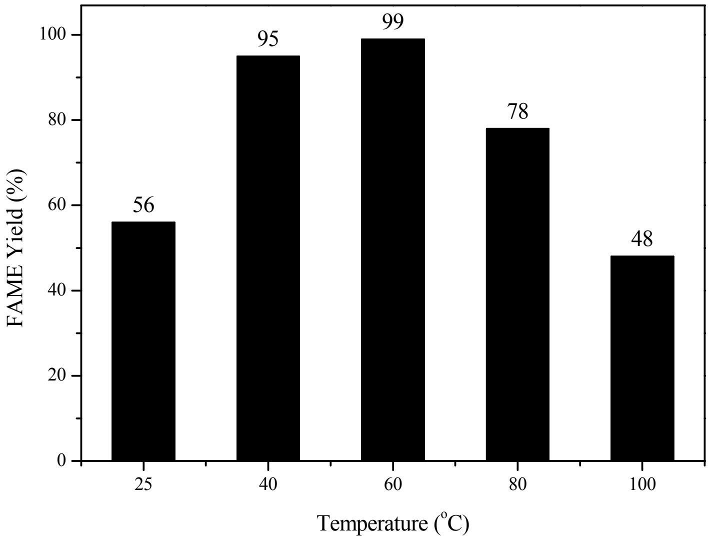

Fig. 1 Influence of the temperature on the biodiesel yield[a].

[a]Reaction conditions: triolein, 0.5 mmol, 443 mg; [Choline][H_2PO_4], 100 w%, 443 mg; lipase, 5 w%, 22 mg; methanol/oil molar ratio,10:1; 24 h.

Influence of the reaction time on the biodiesel production

The effect of the reaction time on catalysis performance was investigated at 60°C and the results were illustrated in Fig. 2. It can be seen that the yield of biodiesel could reach to 99 % and the further extension of reaction time had no significant influence. So, 12 h was chosen as the optimal reaction time for the subsequent studies.

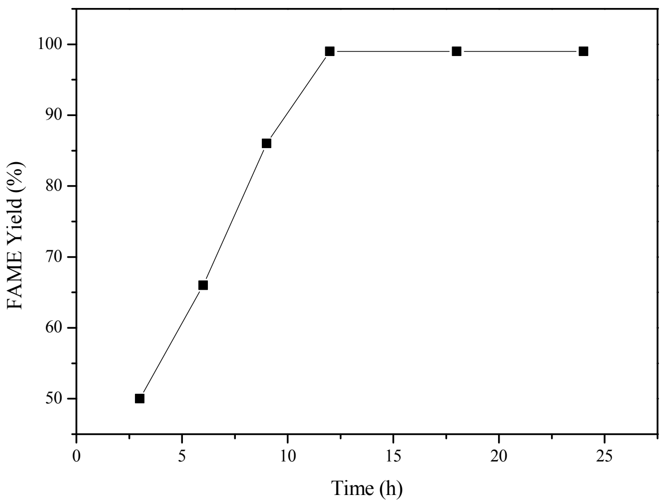

Fig. 2 Influence of the reaction time on the biodiesel yield[a]

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[a]Reaction conditions: triolein, 0.5 mmol, 443 mg; [Choline][H_2PO_4], 100 w%, 443 mg; lipase, 5w%, 22 mg; methanol/oil molar ratio,10:1; temperature 60 °C.

Influence of IL dosage on the biodiesel production

The influence of the dosage of ionic liquid on biodiesel production was investigated using the optimal ionic liquid at 60 °C and the results were collected in Table 2. It can be seen that when weight ratio of the IL to triolein was 50 %, the target biodiesel could reach 99 % yield within 12 h. Therefore, 50 wt% choline dihydrogen phosphate based on triolein weight was optimized for the further investigation.

Table 2. Influence of the dosage of IL on the biodiesel yield

Entry[a]	Ratio/%	Yield[b]/%
1	10	49
2	20	90
3	50	99
4	100	99

[a]Reaction conditions: triolein, 0.5 mmol, 443 mg; [Choline][H_2PO_4]; lipase, 5 w%, 22 mg; methanol/oil molar ratio, 10:1; temperature 60 °C; reaction time, 12 h. [b]GC yield.

Influence of the methanol/oil molar ratio on the biodiesel yield

In the enzymatic production of biodiesel, the molar ratio of methanol/oil had a great influence on the yield of biodiesel. It is evident from Table 3 that the biodiesel yield could reach the highest level when the methanol/oil molar ratio was 10:1 under the optimized reaction temperature and ionic liquid dosage, but the further increase of molar ratio of methanol to oil decreased the yield.

Table 3. Influence of the methanol/oil molar ratio (M: O) on the biodiesel yield

Entry[a]	M: O	Yield[b]/%
1	4:1	85
2	6:1	77
3	8:1	93
4	10:1	99
5	12:1	94
6	14:1	83

[a]Reaction conditions: triolein, 0.5 mmol, 443 mg; [Choline][H_2PO_4], 50 w%, 222 mg; lipase, 5 w%, 22 mg; methanol; temperature 60 °C; reaction time, 12 h. [b]GC yield.

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Influence of the lipase dosage on the biodiesel production

The influence of the dosage of lipase on the biodiesel production was also investigated and the results were collected in Table 4. It is shown that the maxium yield was reached at the weight ratio of 5 % (lipase to triolein).

Table 4. Influence of the lipase dosage on the biodiesel yield

Entry[a]	Ratio/%	Yield[b]/%
1	0	0
2	1	40
3	2	90
4	3	97
5	5	99
6	7	76
7	10	85
8	15	85

[a]Reaction conditions: triolein, 0.5 mmol, 443 mg; [Choline][H_2PO_4], 50 w%, 222 mg; lipase; methanol/oil molar ratio, 10:1; temperature 60 °C; reaction time, 12 h. [b]GC yield.

Influence of the water content on the biodiesel yield

After drying, water content in the ionic liquid [Choline][H_2PO_4] was 0.2 %, as detected by Karl-Fisher titration. So, the influence of water content on the reaction was investigated in the range from 0.2 % to 5.2 % using the optimized ionic liquid [Choline][H_2PO_4], and the results were collected in Table 5. It was shown that the enzymatic effect had no significant variation within the water content of 0.2-3.2 %, suggesting that this reaction system had great tolerance to water or moisture.

Table 5 Influence of the water content on the biodiesel yield

Entry[a]	Ratio/%	Yield[b]/%
1	0.2	99
2	1.2	98
3	3.2	98
4	5.2	85

[a]Reaction conditions: triolein, 0.5 mmol, 443 mg; [Choline][H_2PO_4], 50 w%, 222 mg; lipase, 5 w%, 22 mg; methanol/oil molar ratio, 10:1; temperature 60 °C; reaction time, 12 h. [b]GC yield.

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Reutilization of [Choline][H_2PO_4] and lipase

Considering the relative high cost of enzyme, it is intriguing to reutilize it with simple operation. In our reaction systems, the biodiesel could reach almost quantitative yield under the optimized reaction conditions. Meanwhile, due to the hydrophilic property, the ionic liquid could be automatically separated with the produced biodiesel after the completion of reaction. After simple centrifugation and decantation, the ionic liquid and Novozym 435 could be recovered as a whole, after dried separately they can be used for the next cycle after washing and drying. The reutilization results in Fig. 3 suggested that both the ionic liquid and Novozym 435 could be reused with high catalytic activity. With the adequate prolonged reaction time to 17 h, the yield of biodiesel could still reach to 99 % at the 6th cycles.

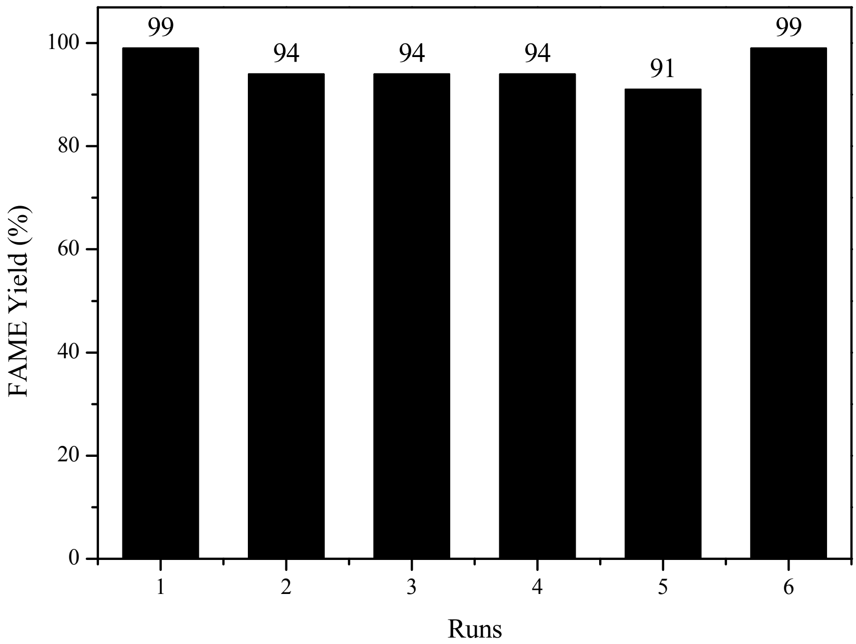

Fig. 3 Reutilization of [Choline][H_2PO_4] and lipase

Reaction conditions: triolein, 0.5 mmol, 443 mg; [Choline][H_2PO_4], 50 w%, 222 mg; lipase, 5 w%, 22 mg; methanol/oil molar ratio, 10:1; temperature 60 ◦ C; reaction time, runs 1-5 were 12 h and run 6 was 17h.

Utilization of oils from different resources

Based on the excellent performance of this enzymatic production system, its utilization in the biodiesel production from different oils like edible sunflower oil and non-edible castor oil was also investigated and the results were collected in Fig.4 and

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Table 6. It is clearly indicated that by using these two types of oils, biodiesel yield was as high as 96 % as calculated by equation 1. This result suggests that this reaction system is great intriguing in promoting biodiesel production from various oil resources.

$$\text{Biodiesel yield} = \frac{\text{total weight of FAME}}{\text{total weight of sunflower oil or castor oil}} \times 100\% \quad (1)$$

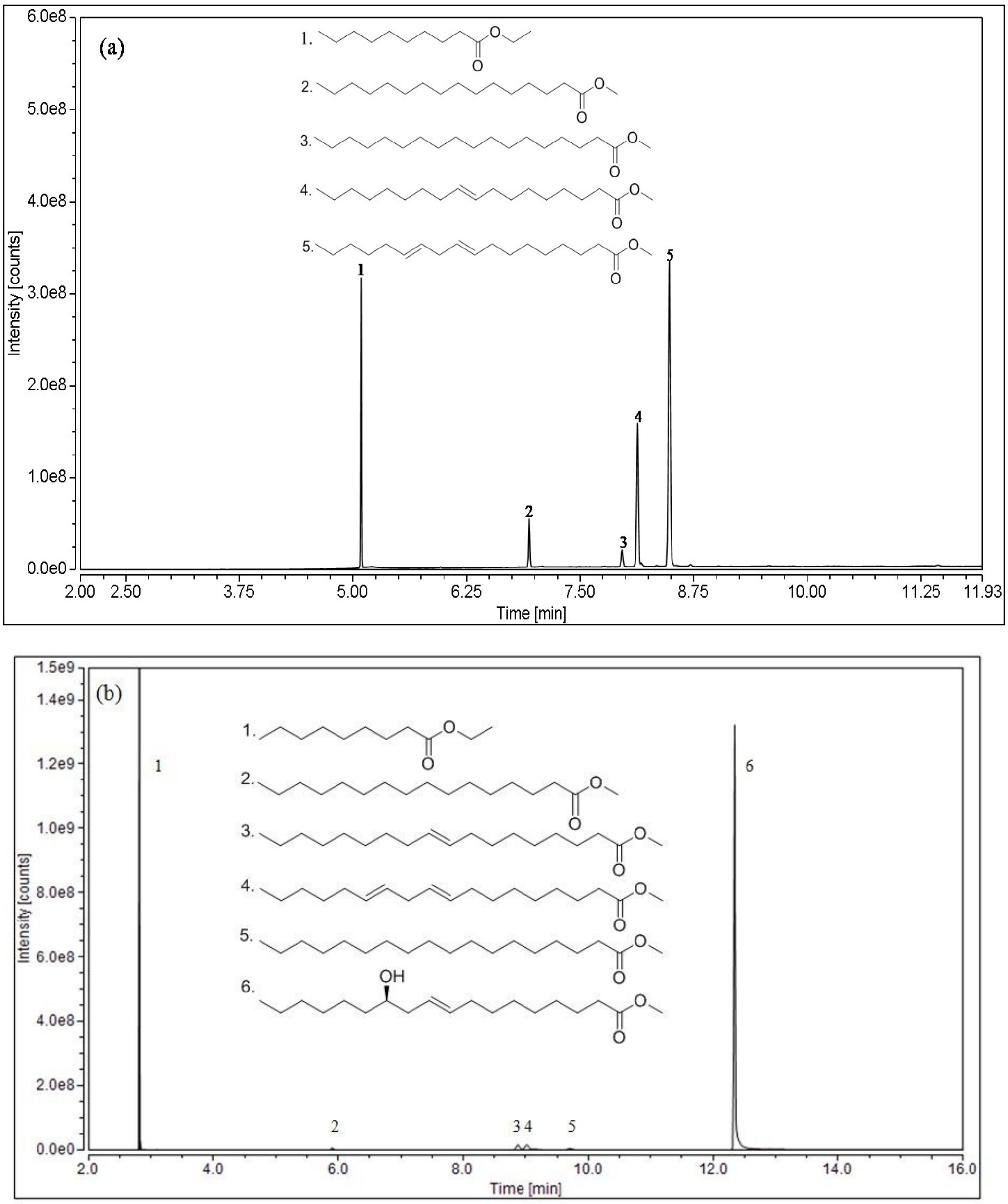

Fig. 4 The gas phase diagram of sunflower oil (a) and castor oil (b)

Table 6. Utilization of oils from different resources

Composition	FAME	FAME Yield/%	
		sunflower oil	castor oil
C16:0	methyl palmitate	5	0.6

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C18:0	methyl stearate	2	0.4
C18:1	methyl oleate	36	1
C18:2	methyl linoleate	53	2
C18:1	methyl ricinoleate	-	92
Total		96	96

[a]Reaction conditions: sunflower oil or castor oil, 0.5 mmol, 443 mg; [Choline][H_2PO_4], 50 w%, 222 mg; lipase, 5 w%, 22 mg; methanol/oil molar ratio, 10:1; temperature 60 °C; reaction time, 12 h. [b]GC yield.

Scale-up experiment

In view of the excellent catalytic activity, simple separation procedure, reusability of the ionic liquid and enzyme, this reaction system has great potential for industrial applications. So, the scale-up experiment to multi-gram scale using non-edible castor oil as resource was also investigated and the results showed that the target biodiesel yield could reach 92 % under the optimized conditions. After the reaction was completed, the biodiesel could be separated from ionic liquids and enzymes automatically as an insoluble phase (Fig. 5), which made it possible to feasibly separate biodiesel product from the catalytic system and reutilize the ionic liquid and enzyme simply and efficiently. These results further supported the potential of this catalytic system in industrial applications.

a. In reaction　　b. After reaction　　c. Biodiesel product

Fig.5 Pictures of biodiesel production process

(a. in reaction, the mixture was stirred under magnetic stirring; b. after the reaction, two phases were formed and the biodiesel was on the upper layer; c. after centrifugation, the isolated biodiesel product.)

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Conclusions

The studies in this work revealed the influences of a series of ionic liquids on the enzymatic production of biodiesel. The results showed that a biocompatible hydrophilic ionic liquid [Choline][H_2PO_4] could efficiently trigger a cleaner biodiesel production procedure. Various oil resources including triolein, sunflower oil and even castor oil could be efficiently converted to biodiesel with simplified operation and work-up procedures, because the hydrophilicity of ionic liquid could lead to the biodiesel production systems conducted at homogeneous reaction and heterogeneous separation. After the completion of the transformation, both of the ionic liquid and enzyme could be recovered and reused for at least five cycles without significant activity decrease, and the reaction system could be easily scaled up to multigram. All of the above made this enzymatic biodiesel production procedure meet the criteria of green chemistry and promising for the further industrial applications. In addition, this work also gave a successful example for the utilization of hydrophilic ionic liquid in enzymatic transesterifications, which will indicate a new viewpoint on the influence of ionic liquids on the activity of enzyme.

Acknowledgements

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Graphic Abstracts

A biocompatible and hydrophilic ionic liquid was found to efficiently promote the enzymatic production of biodiesel with excellent yields from different oil resources. This ionic liquid is easy to be prepared from cheap raw materials. This reaction system was easy to be conducted, the work-up procedure was very simple, and both the ionic liquid and enzyme could be reused for at least five cycles without significant activity decrease.